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# Rapid Quantification of Monoclonal Antibody Titer in Cell Culture Harvests by Antibody-Induced Z-ELP-E2 Nanoparticle Cross-Linking

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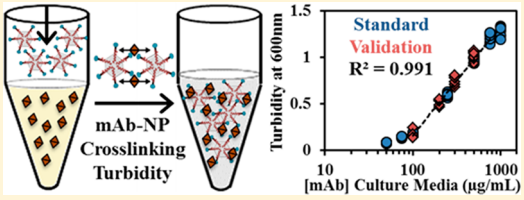
Supporting Information

**ABSTRACT:** Existing assays for the quantification of monoclonal antibody (mAb) cell culture titer often require expensive instruments or reagents and may be limited by the low-throughput or tedious protocols. Here, we developed a quick and cost-effective alternative assay based on mAb-induced cross-linking with Z-domain-ELP-E2 nanocages functionalized by SpyTag/SpyCatcher conjugation. After mixing mAb samples with a fixed nanoparticle concentration for 10 min, we found that the turbidity, measured by absorbance at 600 nm, exhibited a high-signal-to-background ratio and was proportional to the mAb concentration. A simple logarithmic regression was found to fit ($R^2$ = 0.99) the turbidity data for mAb concentrations between 100 and 1000 $\mu$g/mL. The optimized assay procedure was validated using two industrial mAb cell culture harvests, and a bridging study using Octet biolayer interferometry with Protein A sensors confirmed accurate and reproducible results. The assay procedure can be easily adapted to a high-throughput format for rapid mAb titer screening.






With over 50 approved products and several hundreds more currently in clinical development, the market for monoclonal antibodies (mAbs) is expected to exceed $125 billion within the next few years.[1] To capitalize on this rapid growth, process manufacturing platforms have been established to reduce the time required during clinical development.[2] High-throughput, streamlined approaches[3] have enabled the advancement from gene discovery to investigational new drug approval in less than one year.[4] In this early stage of development, mAb-secreting CHO cell lines are screened in bioreactors and optimized for high productivity and quality.[5] Cell line clone selection involves the generation of numerous samples for analytical characterization of key attributes such as mAb titer, glycosylation, charge variants, aggregation, and sequence heterogeneity.[6] To quantify the mAb titer directly from cell culture media with contaminating host cell impurities, techniques such as HPLC-based analytical protein A chromatography,[7] enzyme-linked immunosorbent assay (ELISA),[8] biolayer interferometry (BLI) using ForteBio's Octet instrument,[9] or agglutination-based assays[10] have been reported. However, these methods typically require expensive equipment or reagents or have limitations in throughput, sensitivity, and/or ease of use. Ideally, a simple, high-throughput mAb quantification assay that can be performed without specialized equipment or costly reagents can greatly streamline the transition of new mAbs from discovery into clinical applications.

Target-induced changes in solution turbidity caused by the formation of large aggregates is easy to measure and can be processed in a high-throughput manner using a microplate reader.[11] This low-cost assay format has been used to study protein interactions and aggregation behaviors[12] and is an ideal strategy for simple quantification.[13] Our group has recently developed a new technology for mAb purification based on affinity precipitation using Z-domain-elastin-like polypeptide functionalized E2 (Z-ELP-E2) nanoparticles.[14] Sixty Z-ELP affinity ligands were conjugated to 60 self-assembled subunits of the E2 nanocage[15] using the SpyTag/SpyCatcher system[16] to enable uniform functionalization of Z-ELP-E2 nanoparticles with 100% conjugation (Figure 1A).[17] Because two Z-domain from different nanoparticles can bind to one antibody, multivalent mAb-Z-ELP-E2 interactions rapidly triggered cross-linking into large, insoluble aggregates that resulted in high solution turbidy.[18,19] We speculated that changes in turbidity arising from mAb-induced E2 nanoparticle cross-linking may be proportional to the mAb concentration. The relationship between cross-linking agent concentration and aggregate size or solution turbidity has been well-documented for polymeric nanoparticle systems,[20,21] however, they lack the uniformity in nanoparticle modifications for consistent analytical measurements.

In this paper, we developed a simple, turbidity-based assay for the quantification of industrial mAbs from cell culture samples based on mAb-induced E2 nanoparticle cross-linking. The assay protocol was investigated using two model industrial mAbs with different physical properties. The optimized analytical method can rapidly detect mAb titers of 0.1 to 1.0 g/L within 10−15 min and can be easily adapted to a high-throughput format (Figure 1B). After analysis, the captured mAbs can be purified and eluted for other quality control measurements.

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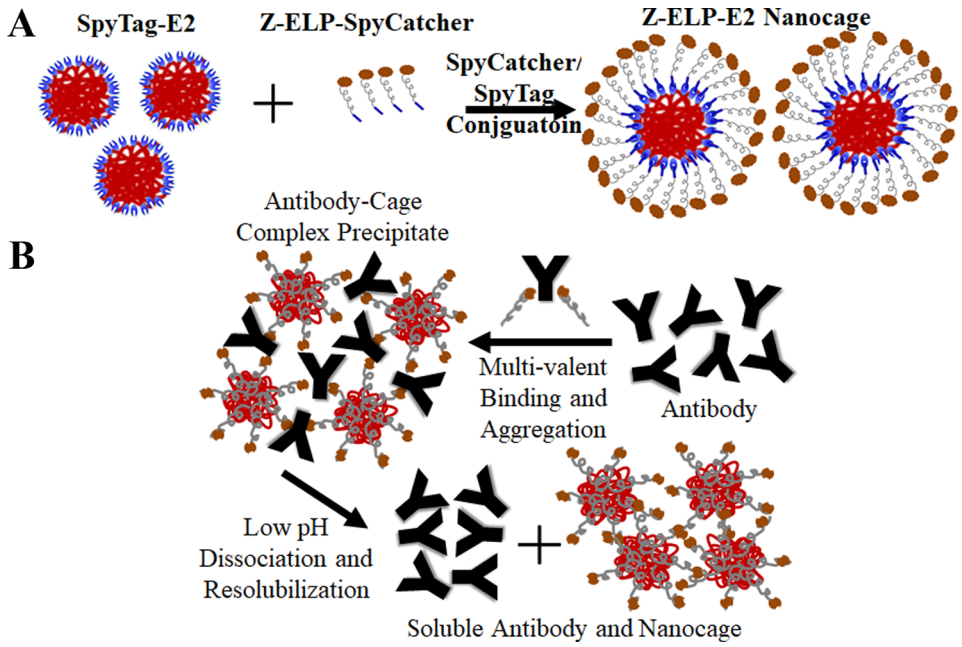


**Figure 1.** (A) Efficient ligation of Z-ELP-SpyCatcher to SpyTag E2 nanocages. 100% ligation can be obtained in 1 h. (B) Procedure for measuring mAb−nanocage crosslinking turbidity and optional mAb purification.

## EXPERIMENTAL SECTION

**Materials.** An *Escherichia coli* strain BLR(DE3) containing a pET24(a) vector encoding for Z-ELP[$KV_8F$-80]-SpyCatcher and an *E. coli* strain BL21(DE3) containing a pET11(a) vector encoding for SpyTag-E2(158) were constructed and described previously.[17] Two purified or clarified cell culture mAbs (mAb A and mAb B) were received as a gift from Bristol-Myers Squibb (BMS) (New York City, NY). mAb cell culture titer and host cell protein (HCP) content were determined previously.[19] Bacto tryptone and yeast extract were purchased from BD Biosciences (Franklin Lakes, NJ). Kanamycin, ampicillin, isopropyl-$\beta$-D-thiogalactoside (IPTG), Cellgro DMEM + 4.5 g/L glucose + L-glutamine +25 mM HEPES media, and 96-well 200uL conical PCR plates were purchased from Fisher Scientific (Pittsburgh, PA). Sodium hydroxide, sodium phosphate, citric acid, ammonium sulfate, sodium chloride, glycine, and a human polyclonal IgG were purchased from Sigma-Aldrich (St. Louis, MO). 96-well half area UV-transparent plates and 96-well solid black microplates were purchased from Corning (Corning, NY). Octet Dip and Read ProA biosensors were purchased from Pall ForteBio (Menlo Park, CA).

**Protein Expression, Purification, and Conjugation.** Z-$ELP_{80}$-SpyCatcher was expressed in BLR(DE3) *E. coli* grown in Terrific Broth (TB) with 50 $\mu$g/mL kanamycin at 37 °C and 250 rpm for 24 h with leaky expression, and SpyTag-E2 was expressed in BL21(DE3) *E. coli* grown in Luria−Bertani Medium (LB) with 100 $\mu$g/mL ampicillin at 37 °C and 250 rpm until induction at an OD600 of 0.5 with 0.2 mM IPTG at 20 °C for 20 h. Cells were harvested and sonicated, and Z-$ELP_{80}$-SpyCatcher was purified by inverse transition cycling (ITC)[22] with 0.5 M ammonium sulfate, and SpyTag-E2 was partially purified by incubating at 70 °C for 10 min, as described previously.[17] 50 $\mu$M purified Z-$ELP_{80}$-SpyCatcher was mixed with 50 $\mu$M SpyTag-E2 in phosphate buffered saline (PBS, 25 mM sodium phosphate, 150 mM sodium chloride, pH 7.2) for 1 h at 20 °C followed by one ITC cycle into PBS, concentrating to 500 $\mu$M Z-ELP-E2 (20× stock solution). 100% Z-$ELP_{80}$-Spy-E2 ligation density was confirmed by 10% acrylamide SDS-PAGE.

**Turbidity Measurement and Analysis.** All turbidity measurements were performed using a Synergy plate reader from BioTek (Winooski, VT) and run at 23 °C. mAb samples were prepared with 25 $\mu$M nanocage at 23 °C in 96-well 200uL conical PCR plates and mixed on a shake plate. Before measurement of absorbance, samples were well mixed using a multichannel pipet by pipetting up and down. Then, 100 $\mu$L was added to a half-area 96-well UV transparent plate, and absorbance was measured at 600 nm. The absorbance value was buffer subtracted and corrected for path length to 1 cm. Standard samples were prepared with purified mAb diluted in cell culture media, and validation samples were prepared with mAb cell culture harvests diluted in media. For ease of analysis, standard samples in the linear range were fit to the equation $Abs600 = m^*\ln[mAb] + b$ using the LINEST function in Microsoft Excel, and the regression coefficients were used to estimate [mAb] of the cell culture harvests.

**mAb−nanoparticle Cross-Linking Turbidity.** For cross-linking kinetics, purified mAbs were prepared at 100, 250, 500, and 750 $\mu$g/mL in cell culture media at pH 7.2 and mixed with 25 $\mu$M nanocage for 2 min before measuring turbidity for 15 or 50 min mAb B was repeated with samples prepared in media titrated to pH 5.0 using 1 M citric acid (Figure S1A). For cross-linking turbidity validation, mAb standards were prepared from 50−1000 $\mu$g/mL using purified mAb, and validation samples were prepared from cell culture harvest at 100, 200, 300, and 500 $\mu$g/mL and mixed with 25 $\mu$M nanocage for 10 min. Six replicate experiments were run for each mAb, and logarithmic regression was performed using standard samples from 100 to 750 $\mu$g/mL. The validation samples were evaluated by the model and the mean and prediction error were calculated. All error bars represent 95% confidence intervals.



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**Octet Bridging Study.** Octet experiments were performed on an Octet RED96e system. Eight ProA biosensors were presoaked in media for 10 min prior to measurement. 200 $\mu$L samples were prepared in a black 96-well plate with mAb standards diluted in media from 5−750 $\mu$g/uL and validation cell culture samples diluted in media at 100, 200, 300, and 500 $\mu$g/uL according to the layout shown in Figure S5. mAb binding was measured every 0.2 s for 60 s, and the sensors were regenerated (glycine, pH 1.5) and neutralized (media) three times between each set of 8 samples. The full data set was the repeated twice more. The binding data was analyzed by the Octet data analysis software, and the initial binding rate of the standard samples was fit to a four-parameter dose response model. The validation samples were evaluated using the fitted regression coefficients.

**mAb Purification after Turbidity Measurement.** The mAb sample can be purified after turbidity measurement using simple washing and elution steps, as described previously.[19] Briefly, the insoluble mAb−nanocage complex was centrifuged, and the pellet was washed with PBS and/or 25 mM sodium citrate pH 5.0. The pellet was resuspended in elution buffer (50 mM sodium citrate pH 3.5), and the purified mAbs were collected in the supernatant after a selective nanoparticle precipitation using 0.25 M ammonium sulfate. The nanoparticles may be regenerated and recycled back into PBS by ITC.

## RESULTS AND DISCUSSION

**Development of a Turbidity Assay for mAb Titers.** Previous studies from our group have demonstrated the rapid and spontaneous cross-linking of Z-ELP-functionalized E2 nanoparticles into large aggregates after mixing with industrial mAb samples.[18] We hypothesized that the resulting increase in solution turbidity correlated with the mAb titer in cell culture samples and could be quantified using a simple microplate format procedure (Figure 1). We investigated this feasibility using two-model industrial mAbs with different properties such as IgG subclass, isoelectric point, cell culture titer, and host cell protein (HCP) content in order to show the generality of the approach (Table S1).

Although sample turbidity is commonly analyzed by measuring absorbance at wavelengths ranging from 350 to 700 nm, the optimal assay wavelength should specifically detect the light scattering from large mAb−E2 nanoparticle aggregates with minimum interference from other solution components.[23] The background signal for different solution components (purified mAb A in PBS, purified nanocages in PBS, and clarified mAb A in cell culture medium) was determined by measuring their absorbance from 240 to 600 nm and compared with mAb−Z-ELP-E2 aggregates in both culture medium and PBS (Figure 2A). A high background was observed for cell culture components for wavelengths less than 420 nm, likely due to host cell debris or absorbing chemicals in the medium. The spectra for mAb−nanoparticle aggregates were significantly higher at wavelengths greater than 500 nm, indicating minimal matrix interference. In this regime, the signal to background ratio was 50:1 and 600 nm was selected as the optimal detection wavelength for the assay (Figure 2B).

To investigate the effect of mAb A concentration on cross-linking turbidity, different concentrations of pure mAb A were mixed with 25 $\mu$M Z-ELP-E2 in PBS at 23 °C, and the absorbance was measured at 600 nm after 30 min. E2 nanoparticles with 100% Z-ELP conjugation (60 Z-ELP per 60 E2 nanocage) were used in order to achieve uniformity of the assay and the highest sensitivity.[17] A logarithmic dependence of mAb concentration on absorbance was observed with an overall sigmoidal response on a semilog plot (Figure 2C). For mAb concentrations ≥10 $\mu$M, the aggregated particles began to settle out of solution, as evidenced by a decrease in absorbance and increased variability. In contrast, the solution turbidity exhibited a strong dependence on the mAb concentrations between 0.5 to 10.0 $\mu$M, suggesting that the extent of cross-linking and aggregate size correlated to the mAb concentration in this range. The limit of detection was approximately 200 nM (~0.04 g/L), as minimal mAb−nanoparticle cross-links were formed below this concentration to induce a detectable increase in turbidity.

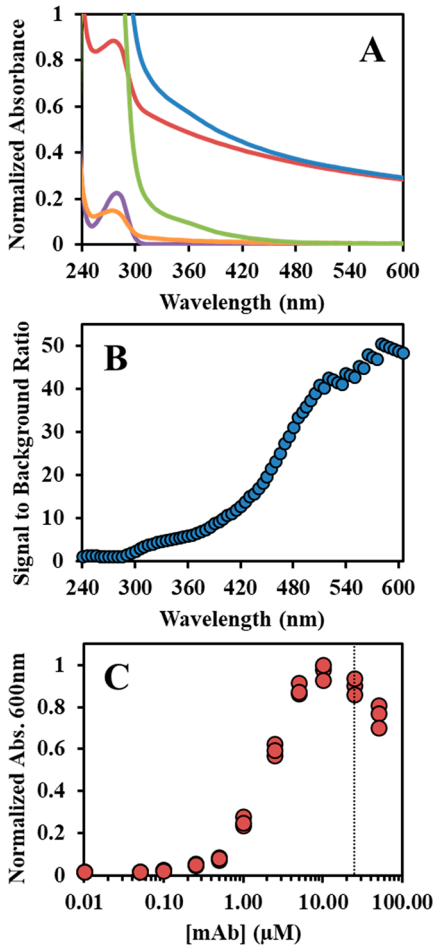


**Figure 2.** Optimization of mAb−nanoparticle cross-linking turbidity measurement. (A) Absorbance spectra for clarified mAb A-nanoparticle mix (blue), purified mAb A-nanocage mix (red), clarified mAb A (green), purified mAb A (purple), and purified E2 nanoparticle (orange). (B) The ratio of absorbance for clarified mAb A-nanoparticle mix to clarified mAb A. (C) The normalized absorbance at 600 nm for purified mAb A from 0.01 to 50 $\mu$M (red circle) mixed with 25 $\mu$M purified nanocage (black line) in triplicate.

**Determination of mAb−Nanoparticle Cross-Linking Kinetics.** The mAb−nanoparticle aggregation kinetics were investigated using purified mAbs (within the linear concentration range from 100 to 750 $\mu$g/mL) to ensure fast and reproducible results. Previous work indicated that mAbs with a basic isoelectric point aggregate immediately upon mixing at neutral pH, while mAbs with a more acidic isoelectric point required titration to pH 5.0 to achieve a similarly fast


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kinetics.[19] In cell culture media at pH 7.2, mAb A (net positive, pI = 8.3) induced cross-linking rapidly and the turbidity value reached a steady state within 10 min of mixing (Figure 3A).

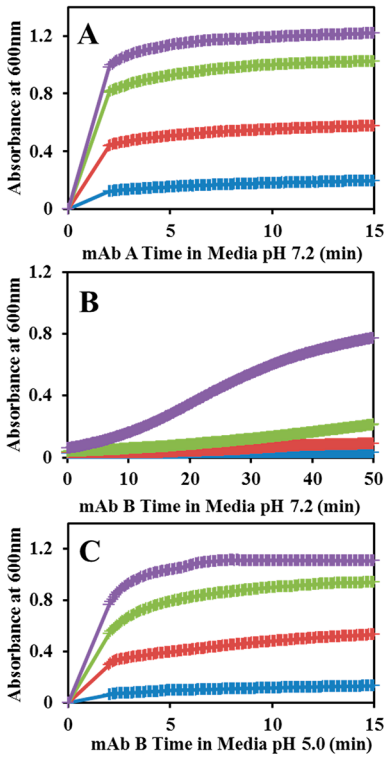


**Figure 3.** mAb−Nanoparticle aggregation kinetics. Changes in turbidity was detected for 750 (purple), 500 (green), 250 (red), or 100 (blue) $\mu$g/mL mAb mixed with 25 $\mu$M nanocage for (A) mAb A in cell culture medium pH 7.2, (B) mAb B in cell culture medium pH 7.2, or (C) mAb B in cell culture medium titrated to pH 5.0.

Although mAb B (pI = 6.8) exhibited slower kinetics at neutral pH (Figure 3B), a simple titration to pH 5.0 using 1 M citric acid restored the fast aggregation (Figure 3C) with the turbidity value reaching a steady state after 15 min. Analysis of the logarithmic regression resulted in a good fit ($R^2$ = 0.99) with consistent regression coefficients after 10−15 min mixing (Figure S2A-B). Quantification of a human polyclonal IgG was also evaluated after mixing for 15 min in sodium citrate, pH 5.0, and a similar good fit was obtained (Figure S1B). For reproducible data, all future turbidity measurements were determined after 15 min, where it is assumed the nanoparticles have cross-linked with all available IgG binding sites. These results indicate that any mAb cell culture sample can be diluted into the detectable range for rapid quantification using the E2 nanoparticle-based cross-linking turbidity assay.

**Validation of the mAb−Nanoparticle Cross-Linking Turbidity Assay.** The mAb−nanoparticle cross-linking turbidity assay was validated using clarified cell culture harvests. Standard curves were first prepared by mixing purified mAbs from 50 to 1000 $\mu$g/mL with 25 $\mu$M fully decorated Z-ELP-E2 nanocages in cell culture media for 10 min before measuring absorbance at 600 nm. A logarithmic regression was performed to fit the six replicate data sets within the linear rage (100−750 $\mu$g/mL) (Figure 4A,B). A good fit ($R^2$ ~ 0.99) was obtained for both mAbs with similar regression coefficients. The resulting standard curves generated

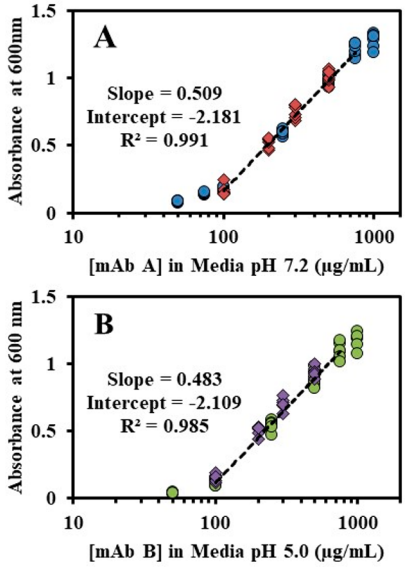


**Figure 4.** Cross-linking turbidity assay validation. (A) mAb A standards (blue circle) prepared with purified mAb and validation using clarified mAb in cell culture medium (red diamond). (B) mAb B standards (green circle) prepared with purified mAb and validation mAb cell culture (purple diamond). All samples were run with 6 replicates. The standards 100−750 $\mu$g/uL were fit to logarithmic function for ease of analysis.

were used to determine mAb titers in clarified cell culture samples.

To validate the assay, clarified mAb samples were diluted to 100, 200, 300, and 500 $\mu$g/mL in cell culture media (pH 7.2) for mAb A or media titrated to pH 5.0 for mAb B. Samples were analyzed by the regression models, and the predicted concentrations were consistent with the expected values (Figure 5A). Higher variabilities were observed at higher mAb concentrations, but the coefficient of variation (CV) was less than 10% for all samples. The prediction error ranged from

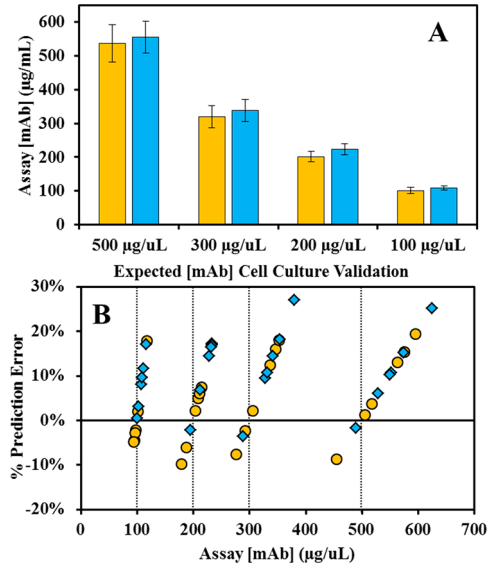


**Figure 5.** Model fit and prediction error. (A) Fitted validation data for mAb A (orange) or mAb B (light blue). Error bars represent 95% confidence intervals. (B) Prediction error for validation fitted data for mAb A (orange circle) or mAb B (light blue diamond).



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−10 to +25%, and on average, the predicted values resulted in a slight overestimation of mAb titer for both mAb A and B (Figure 5B). This may be due to the coprecipitation of host cell protein or media components that also increase the turbidity during the initial mAb−nanoparticle cross-linking. Since the mAb B culture contained significantly less HCP than mAb A (Table S1) and exhibited a higher overall positive prediction error, the main sources of variability are likely due to inefficient mixing and dilution/pipetting errors.

To further validate the accuracy of these results, a bridging study was performed by comparing the cross-linking turbidity assay with an Octet RED96e mAb quantification assay using Protein A sensors purchased from Pall ForteBio. The Octet assay was carried out following standard protocols. Purified mAb standard curves were used to determine mAb A and B cell culture samples prepared at 100, 200, 300, 500 $\mu$g/mL using a four-parameter dose−response model to fit the initial binding rates (Figure S3A-C). The calculated cell culture mAb concentrations measured by Octet were compared to the turbidity assay, and excellent agreements were obtained ($R^2$ > 0.99) for both mAbs. The Octet assay exhibited lower variability at [mAb] < 250 $\mu$g/mL, but the variability was similar to the turbidity assay at higher concentrations. These results confirm that mAb−nanoparticle cross-linking can be used to provide the same accurate determination of mAb titers as the Octet assay without the use of expensive equipment or sensors. In addition, mAbs samples may be collected after measurement and purified in a high-throughput format using established affinity precipitation protocols for other quality control measurements. As an example, a 1 mg/mL mAb cell culture sample was captured by 25 $\mu$M Z-ELP-E2, and after the turbidity measurement, the mAb−nanocage complex was pelleted, washed, and resuspended with high mAb elution yield and purity (Figure S4). There was no detectable leaching of the nanoparticle into the mAb elution supernatant by SDS-PAGE analysis. These purified samples can be submitted directly or buffer exchanged for additional analytical characterization. This may be especially beneficial for assays that require purified protein such as glycosylation analysis.[24] The Z-ELP-E2 nanoparticles can also be regenerated for future testing (Figure 6).

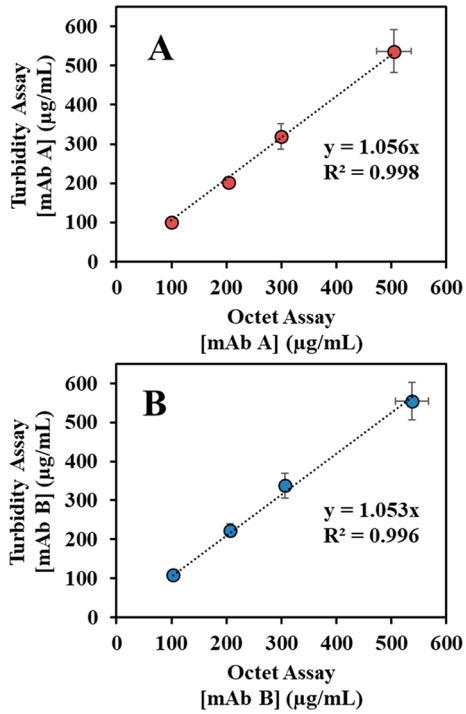


**Figure 6.** Comparison of turbidity assay and Octet assay for validation samples of mAb cell culture harvests for mAb A (A) and mAb B (B). Error bars represent 95% confidence intervals.

## CONCLUSIONS

With a surplus of mAb candidates currently in clinical development, a high-throughput, cost-effective assay for mAb titer is beneficial to accommodate the numerous samples produced from early upstream process optimization. Existing assays require expensive, specialized equipment and may be limited by throughput or sensitivity. In the current study, we developed a new cost-effective method for measuring mAb cell culture titers using a simple concentration-dependent E2 nanoparticle cross-linking turbidity assay. The most unique aspect of our Z2 scaffold is the ability to generate nanoparticles containing 60 Z-ELP-80-SpyCatcher due to the ability to achieve 100% ligation efficiency.[17] The rapid SpyTag-SpyCatcher covalent bond formation allows for a simple and quick method to create highly uniform functionalized E2 nanoparticles for the assay. Other than the two *E. coli* fermentations required for the recombinant expression of Z-ELP and E2, there are minimal capital or material costs associated with the production of the nanoparticles.[22] ITC is used for purification from *E. coli* lysate, and equimolar mixing of the two components is all that is required for conjugation. More importantly, the Z-ELP-E2 nanoparticles can be regenerated for subsequent assays, as demonstrated previously.[18]

After mixing the Z-ELP-E2 nanoparticles with a mAb sample, the rapid increase in turbidity was analyzed using a spectroscopic measurement of absorbance at 600 nm. This enabled the specific detection of mAb-induced aggregates with a 50-fold signal enhancement over the background cell culture media. A logarithmic dependence of mAb concentration and cross-linking turbidity was observed in the range of 100−1000 $\mu$g/mL. The optimized turbidity assay was validated using mAb cell culture samples with known concentrations and compared to an Octet assay using Protein A sensors. Excellent agreement was obtained between the two methods.

The reported mAb−nanoparticle cross-linking turbidity assay is particularly useful for antibody-secreting cell line development and clone selection. A simple 10 min mixing step and absorbance measurement can quantify the mAb titers, sufficient for rapid screening purposes. Future work will investigate the detection of other target analytes through nanoparticle cross-linking turbidity by conjugating interchangeable affinity domain-ELP-SpyCatcher fusion proteins to the SpyTag-E2 scaffold.

## ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.8b04083.

Supporting figures and tables on mAb properties, turbidity measurements, Octet mAb titer measurement and validation, and SDS-PAGE of mAb purification (PDF)



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### Notes

The authors declare no competing financial interest.


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